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Methods in Enzymology

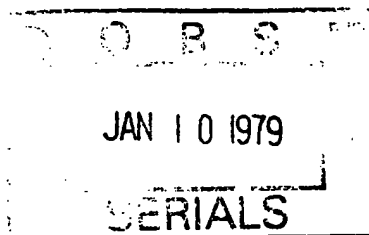
Volume LVII

*Bioluminescence and
Chemiluminescence*

EDITED BY

Marlene A. DeLuca

DEPARTMENT OF CHEMISTRY
UNIVERSITY OF CALIFORNIA AT SAN DIEGO
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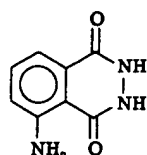
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[36] The Chemiluminescence of Luminol and Related Hydrazides

By DAVID F. ROSWELL and EMIL H. WHITE

Chemiluminescence is the production of light by chemical reactions. Electronically excited states are formed in the chemical reaction, and light emission from these completes the process. The relationship between chemiluminescence and absorption/emission phenomena, such as fluorescence, are shown in Fig. 1.

A surprisingly large number of chemical reactions are chemiluminescent.¹⁻⁴ This paper, however, will deal only with a rather famous example, the chemiluminescence of luminol [5-amino-2,3-dihydrophthalazine-1,4-dione (compound I)]; some derivatives and analogs will also be discussed.



(I)

The first report of chemiluminescence from luminol was made by Albrecht⁵ in 1928. Since that time the reaction of luminol and other derivatives of the general hydrazide structure⁶ have been studied extensively. A number of reviews on this subject have appeared over the last 15 years,⁷⁻¹⁰ and hence the historical aspects of the problem will be treated only briefly.

¹ K.-D. Gundermann, *Top. Curr. Chem.* **46**, 61 (1974).

² E. H. White and D. F. Roswell, *Acc. Chem. Res.* **3**, 54 (1970).

³ M. J. Cormier, D. M. Hercules, and J. Lee, eds., "Chemiluminescence and Bioluminescence." Plenum, New York, 1973.

⁴ K.-D. Gundermann, "Chemilumineszenz Organischer Verbindungen." Springer-Verlag, Berlin and New York, 1968.

⁵ H. O. Albrecht, *Z. Phys. Chem.* **136**, 321 (1928).

⁶ Luminol as well as being named as a phthalazinedione is also referred to as 3-aminophthalic hydrazide.

⁷ E. H. White, in "Light and Life" (W. D. McElroy and B. Glass, eds.), 1st ed., p. 183. Johns Hopkins Press, Baltimore, Maryland, 1961.

⁸ K.-D. Gundermann, *Angew. Chem., Int. Ed.* **4**, 566 (1965).

⁹ F. McCapra, *Quart. Rev. Chem. Soc.*, **20**, 485 (1966).

¹⁰ J. W. Haas, *J. Chem. Educ.* **44**, 396 (1967).

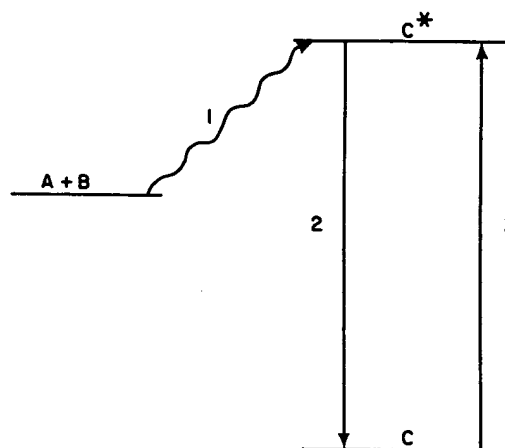
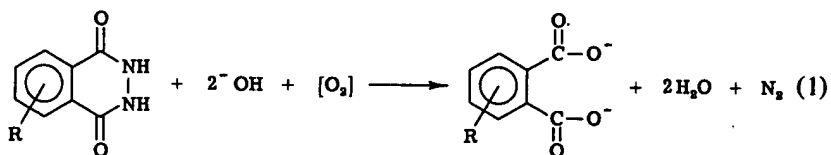


FIG. 1. Chemiluminescence, path 1 + 2; fluorescence, path 2; absorption, path 3.

Reaction Conditions

The chemiluminescent reactions of luminol are all oxidations [Eq. (1)]. A wide variety of reaction conditions have been employed; the reaction can be carried out either in protic media or in aprotic solvents such as dimethyl sulfoxide (DMSO), hexamethylphosphoric acid triamide (HMPT), or dimethylformamide (DMF). In aprotic media, only oxygen and a strong base are required for chemiluminescence.⁷



Water is the most common protic solvent, although the reaction has been studied in the lower alcohols as well. The reaction in these solvents usually requires base, an oxidizing agent, and either oxygen or a peroxide, depending on the system. Hypochlorite, ferricyanide, and persulfate are commonly used oxidizing agents. In place of the oxidizing agents, chelated transition metal complexes, pulse radiolysis,¹¹ or sonic waves¹² have been used in the presence of either oxygen or peroxides.

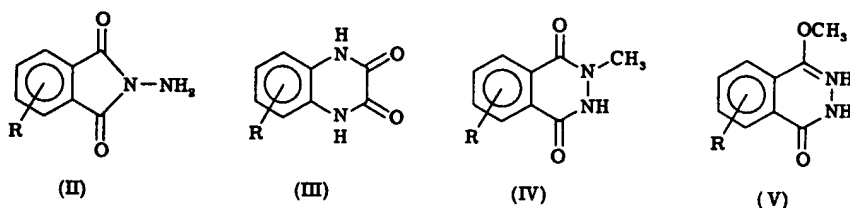
¹¹ J. H. Baxendale, *Chem. Commun.* 1971, 1490 (1971).

¹² E. N. Harvey, *J. Am. Chem. Soc.* 61, 2392 (1939).

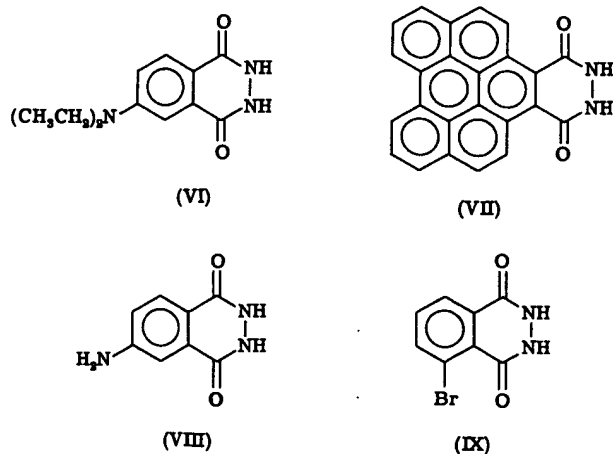
One of the simplest systems involves adding luminol to a solution of hydrogen peroxide in dilute sodium carbonate that has been partially neutralized immediately before the luminol is added.¹³

Structural Effects

Early workers investigated the effect of gross structural modifications of luminol [Eq. (1)].¹⁴⁻¹⁶ Compounds isomeric to (I) in the heterocyclic ring—compounds (II) and (III), as well as N or O methylation products (IV and V, respectively)—were nonchemiluminescent.



Analogues in which the amino group was replaced by other substituents, or analogues with substitution anywhere in the nonheterocyclic ring were generally found to be chemiluminescent. These compounds vary in their light-producing ability; e.g., compounds (VI)¹ and (VII)¹³ are more chemiluminescent than luminol whereas compounds (VIII) and (IX) are of lower efficiency.¹⁴



¹³ E. H. White and R. B. Brundrett, in "Chemiluminescence and Bioluminescence" (M. J. Cormier, D. M. Hercules, and J. Lee, eds.), p. 231. Plenum, New York, 1973.

¹⁴ H. D. K. Drew and F. H. Pearman, *J. Chem. Soc. London* 1937, 586 (1937).

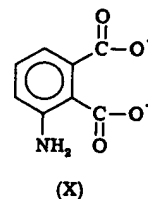
¹⁵ H. D. K. Drew and R. F. Garwood, *J. Chem. Soc. London* 1939, 836 (1939).

¹⁶ E. H. Huntress and J. V. K. Gladding, *J. Am. Chem. Soc.* 64, 2644 (1942).

The chemiluminescence quantum yield (Φ_{ch}), where Φ_{ch} = photons produced/molecules reacted, is used as the measure of light-producing ability. For luminol, Φ_{ch} = 0.01; and for compound (VII), Φ_{ch} = 0.08. This quantum yield may also be defined as $\Phi_{\text{ch}} = \Phi_{\text{R}}\Phi_{\text{ES}}\Phi_{\text{FI}}$, where Φ_{R} is the fraction of reacting molecules following the correct chemical path, Φ_{ES} is the fraction of excited states produced, and Φ_{FI} is the fluorescent quantum yield of the emitter. The light-producing ability of luminol analogs is thus a function of three factors; of these, Φ_{ES} and Φ_{FI} appear to be the most important.^{17,18}

Products

Under the mild reaction conditions possible in aprotic solvents, the organic product of the luminol oxidation is 3-aminophthalate (X), which is



produced almost quantitatively.¹⁹ Nitrogen is evolved and oxygen is consumed in essentially molar amounts.¹⁹ In protic media the reaction conditions are so vigorous that degradation of the initially formed aminophthalate occurs, but the overall reaction is believed to be the same.

The Light Emitter

In both protic and aprotic media the product phthalate ion, (X), is found in an electronically excited state and is responsible for the observed emission. The identity of the fluorescence spectrum of the product with the chemiluminescence emission spectra of the reaction (see Fig. 2) establishes this point. As shown in Fig. 2a, the emission from the oxidation of luminol, (I), in aqueous media occurs at 425 nm and is superimposable on the fluorescence spectra of the total reaction products and also on the spectrum of 3-aminophthalate ion. In aprotic media the emission maxima are shifted to longer wavelengths. The long-wavelength emission has been attributed to the quinonoid tautomer, (XI), of (X). This species would not be present in

¹⁷ R. B. Brundrett, D. F. Roswell, and E. H. White, *J. Am. Chem. Soc.* **94**, 7536 (1972).

¹⁸ R. B. Brundrett and E. H. White, *J. Am. Chem. Soc.*, **96**, 7497 (1974).

¹⁹ E. H. White, O. C. Zafriou, H. M. Kagi, and J. H. M. Hill, *J. Am. Chem. Soc.* **86**, 940 (1964).

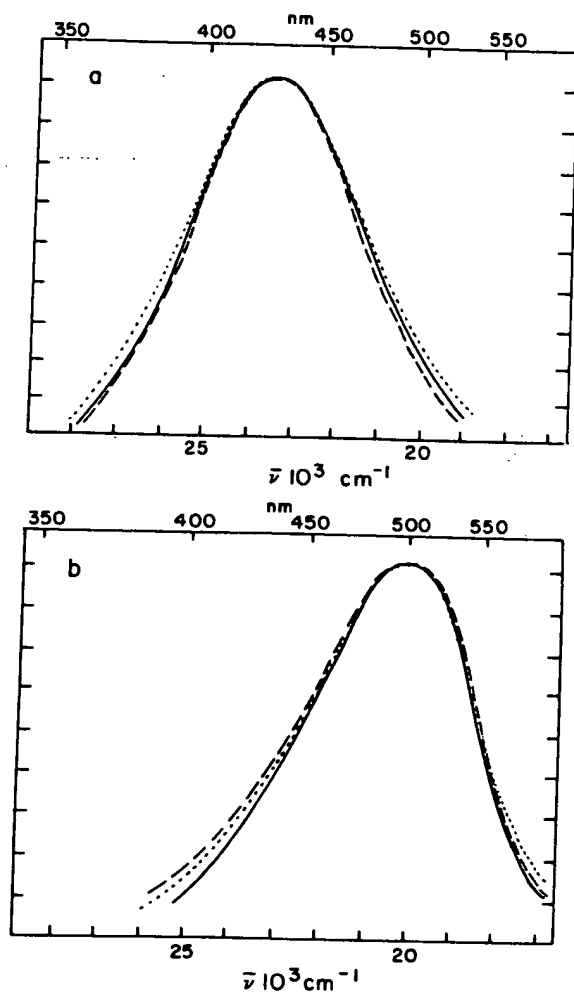
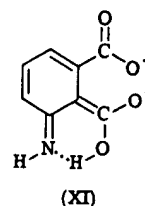


FIG. 2. Fluorescence and chemiluminescence emission spectra: (a) solvent is water; (b) dimethyl sulfoxide. —, chemiluminescence of luminol;, fluorescence of 3-aminophthalate; ----, fluorescence of total reaction mixture. Permission to reprint is granted by the American Chemical Society. *J. Am. Chem. Soc.* 95, 2610 (1973).



highly aqueous media²⁰ because of the lowered basicity of a carboxylate group hydrogen-bonded to water molecules. In mixed solvent systems (DMSO-H₂O) both emissions occur, the 425-nm band increasing with water concentration. These reaction systems show a one-to-one correlation between chemiluminescence and the fluorescence of (X) as long as the base used is a quaternary ammonium hydroxide.

If other bases are used, this correspondence is lost; in a system of 90% dimethyl sulfoxide (DMSO)-water made basic with alkali metal hydroxides (MOH), the shorter-wavelength emission band is more pronounced in 3-aminophthalate fluorescence than in luminol chemiluminescence when $M = \text{Na}^+$ or K^{+20} (Fig. 3).

The fluorescence spectrum of 3-aminophthalate shows a marked increase in the shorter-wavelength emission band with increasing sodium ion concentration, and at high sodium ion concentrations the longer-wavelength band is almost lost. The absorption spectrum of 3-aminophthalate ion shifts with increasing sodium ion concentration, suggesting that there is considerable ion pairing in the ground state. Because of the short lifetime of the excited state, this ion pairing persists in the excited state. The ion pair of aminophthalate ion, (X), would be expected to have a lower electron density at the carbonyl moiety of the carboxylate group, and thus be less able to bring about the intramolecular proton transfer required to form the quinonoid form (XI), which is responsible for the long-wavelength emission. Thus, metal ions form ion pairs with (X), which fluoresce at about 425 nm, whereas the free ion can pass over to the quinonoid form, (XI), which fluoresces at about 510 nm.

The differences in the fluorescence emission spectra of 3-aminophthalate ion and the chemiluminescence of luminol (Fig. 3) can also be explained by the ion-pair effect. The fraction of excited aminophthalate ions in the ion-pair form differs in fluorescence and chemiluminescence. The fraction in fluorescence is essentially determined by the ground-state equilibrium. The fraction of ion pairs in chemiluminescence is determined

²⁰ E. H. White, D. F. Roswell, C. C. Wei, and P. D. Wildes, *J. Am. Chem. Soc.* **94**, 6223 (1972); see also J. Lee and H. H. Seliger, *Photochem. Photobiol.* **15**, 227 (1972) and J. D. Gorsuch and D. M. Hercules, *Photochem. Photobiol.* **15**, 567 (1972).

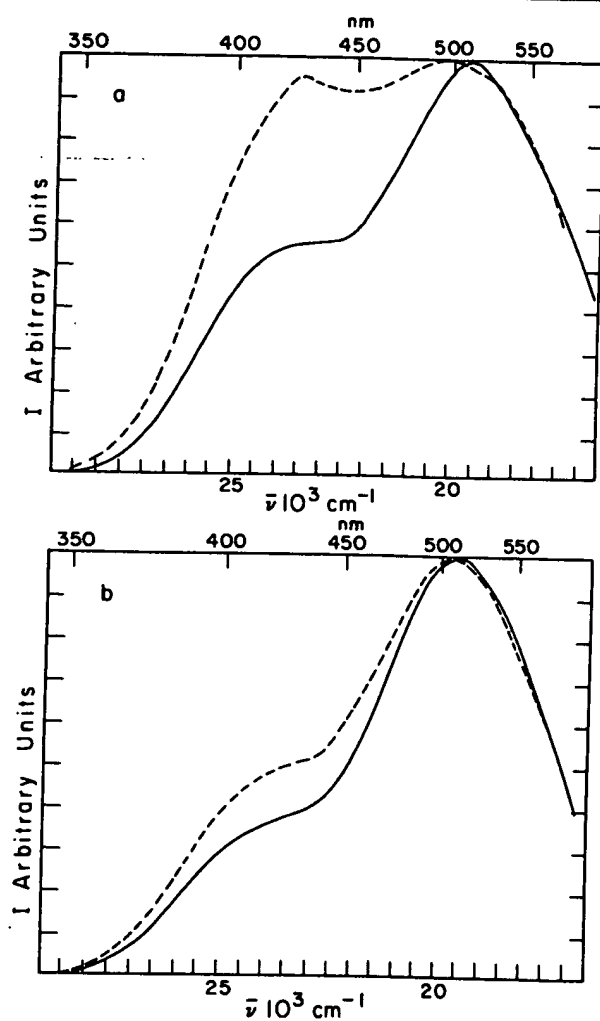
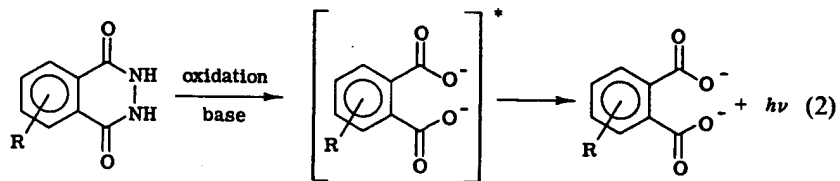


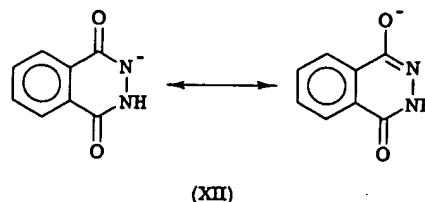
FIG. 3. Luminol chemiluminescence (—) and 3-aminophthalate fluorescence (---) in 30 mol % water, 70 mol % dimethyl sulfoxide with (a) 20 mM sodium hydroxide; (b) 20 mM potassium hydroxide. Permission to reprint is granted by the American Chemical Society. *J. Am. Chem. Soc.* 95, 2610 (1973).

at the transition state of the reaction, and in the present case this value is lower than that in fluorescence. It should be pointed out that the above observations pertain to luminol and that usually the excited states formed in chemiluminescence and fluorescence are the same.

The general picture of the chemiluminescence of cyclic hydrazides—that light emission occurs from the corresponding phthalate ion [Eq. (2)]—appears to hold with only rare exceptions. The parent compound ($R=H$,



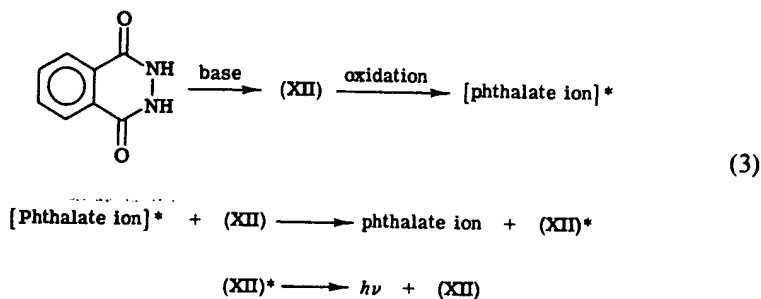
phthalic hydrazide) is essentially nonchemiluminescent in the protic system;²¹ this is not surprising, since phthalate ion under the reaction conditions is not fluorescent. In aprotic solvents such as DMSO, however, the reaction of phthalic hydrazide with base and oxygen does give a yellow emission of moderate intensity, yet under the same conditions phthalate ion is again not fluorescent. The emission spectrum of this chemiluminescence matches the fluorescence spectrum of the monoanion of the starting hydrazide, (XII), under the reaction conditions. Equation (3) has been



proposed to account for this result.²² This scheme requires production of excited phthalate and subsequent intermolecular energy transfer to the anion of the starting material from which emission occurs. The energy transfer apparently occurs between an excited phthalate ion hydrogen bonded to a phthalic hydrazide anion, since added fluorescers without acidic protons, such as acridone or the salt of *N*-methyl phthalic hydrazide, do not accept the excitation energy.

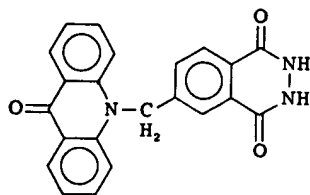
²¹ J. Stauff and G. Hartmann, *Ber. Bunsenges. Phys. Chem.* 69, 145 (1965).

²² E. H. White, D. F. Roswell, and O. C. Zafriou, *J. Org. Chem.* 34, 2462 (1969).



Energy Transfer

Energy transfer has also been shown to occur in the chemiluminescence of hydrazides²³⁻²⁶ which are linked to some highly fluorescent groups. Compound (XIII) is a good example of this type of structure. In the case of



(XIII)

compound (XIII), oxidation of the hydrazide portion of the molecule (in protic or aprotic media) results in emission from the acridone moiety. This emission occurs because of rapid intramolecular singlet-singlet energy transfer from the electronically excited phthalate ion to the acridone portion of the molecule.

Reaction Mechanism

Information about the mechanism of oxidation of luminol and other hydrazides has been obtained in studies using both protic and aprotic media. In aprotic media (DMSO), the dinegative ion, (XIV), formed in a stepwise reaction with base is a crucial intermediate [Eq. (4)].²⁷ Subsequent

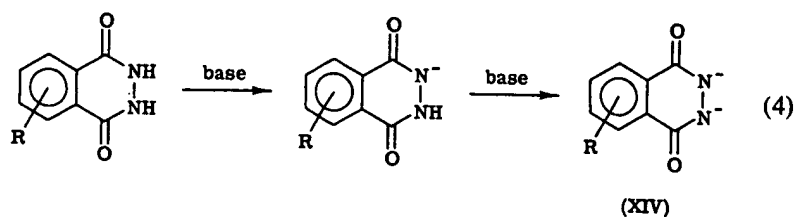
²³ E. H. White and D. F. Roswell, *J. Am. Chem. Soc.* **89**, 3944 (1967).

²⁴ E. H. White, D. R. Roberts, and D. F. Roswell, in "Molecular Luminescence" (E. C. Linn, ed.), 1st ed., p. 479. Benjamin, New York, 1969.

²⁵ D. F. Roswell, V. Paul, and E. H. White, *J. Am. Chem. Soc.* **92**, 4855 (1970).

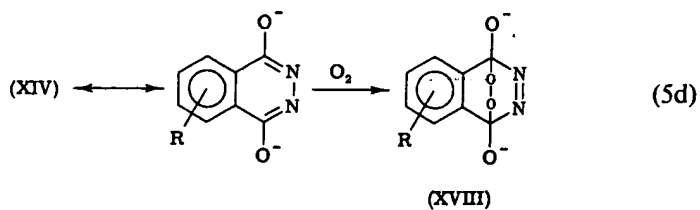
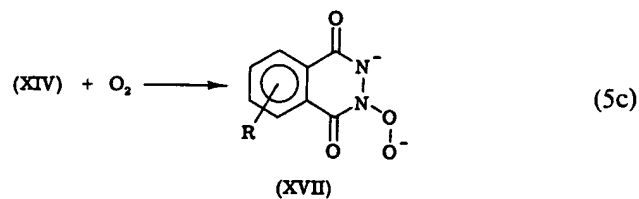
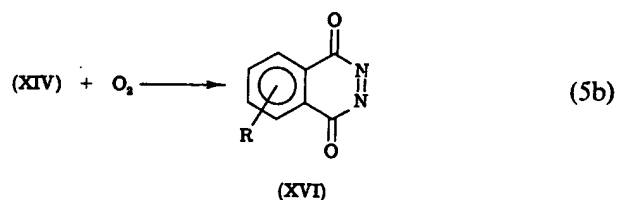
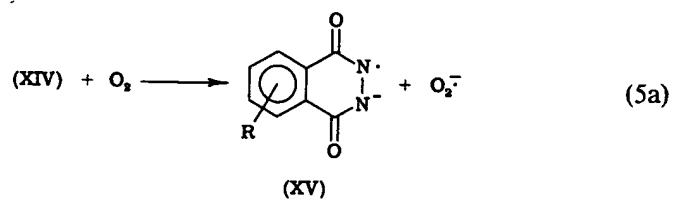
²⁶ D. R. Roberts and E. H. White, *J. Am. Chem. Soc.* **92**, 4861 (1970).

²⁷ E. H. White and M. M. Bursey, *J. Am. Chem. Soc.* **86**, 941 (1964).

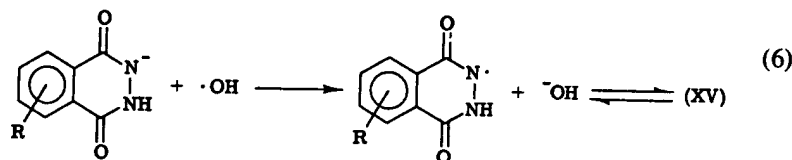


reaction of this intermediate with oxygen via some as yet undetermined pathway produces the phthalate ion in an excited state.

The reaction of the dinegative ion could follow several pathways, as indicated in Eqs. (5a)–(5d).

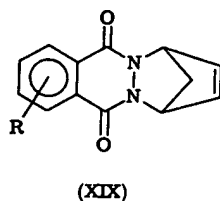


The radical anion intermediate, (XV), in Eq. (5a) has also been proposed for the reaction in protic media when transition metal complexes are used as catalysts, and for the electrochemical oxidation.²⁸ In protic media it could be formed by the pathway shown in Eq. (6).^{7,29} Alternatively, it has



been proposed³⁰ that the metal ion forms a complex with the hydrazide, which is then oxidized, and it has also been suggested that it is a $HO_2^- M^{2+}$ complex which produces the hydrazide radical anion.³¹

The azaquinone proposed in Eq. (5b) has received considerable attention as a possible reaction intermediate; it was proposed as an intermediate in the first paper published on the chemiluminescence of luminol.⁵ Later workers, on the basis of kinetic evidence, have suggested azaquinones as intermediates in the persulfate/hydrogen peroxide-driven chemiluminescence of luminol as well as in the hypochlorite oxidation.^{32,33} Azaquinones have been trapped as their cyclopentadiene adducts,³⁴ (XIX), from solution, which in the absence of the trapping agent normally exhibit



chemiluminescence. These adducts have been identified by thin-layer chromatography; the yield is low, accounting for only a small fraction of the starting hydrazide.

²⁸ B. Epstein and T. Kuwana, *Photochem. Photobiol.* **4**, 1157 (1965).

²⁹ P. B. Shevlin and H. A. Neufeld, *J. Org. Chem.* **35**, 2178 (1970).

³⁰ A. K. Babko and N. M. Lukovskaya, *Ukr. Khim. Zh.* **30**, 508 (1964).

³¹ T. G. Burdo and W. R. Seitz, *Anal. Chem.* **47**, 1639 (1975).

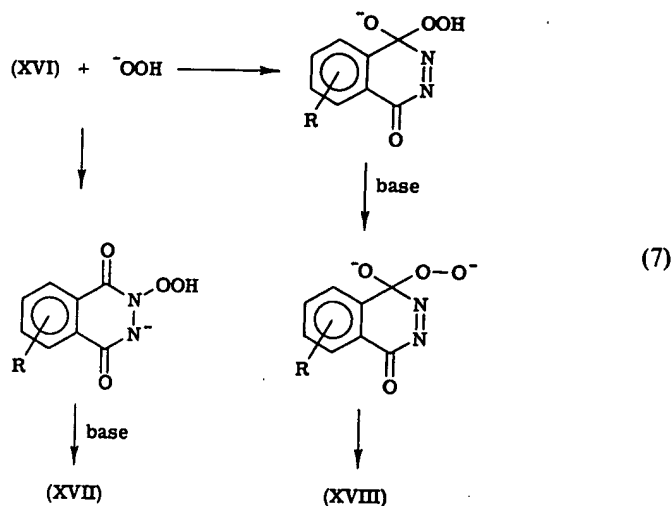
³² M. M. Rauhut, A. M. Semsel, and B. G. Roberts, *J. Org. Chem.* **34**, 2431 (1966).

³³ W. R. Seitz, *J. Phys. Chem.* **79**, 101 (1975).

³⁴ Y. Omote, T. Miyake, and N. Sugiyama, *Bull. Chem. Soc. Jpn.* **40**, 2446 (1967).

Diazaquinones are reactive but isolable compounds.³⁵⁻³⁷ They chemiluminesce in the presence of alkaline hydrogen peroxide and, unlike the hydrazides, do not require peroxide-decomposing catalysts or oxidizing agents. Comparison of the emission spectra from the chemiluminescence of the hydrazide and the corresponding diazaquinone show that the emitter (the phthalate ion) is the same in the two cases. The diazaquinone has been detected by stop-flow spectroscopy during the hypochlorite-catalyzed oxidation of 2,3-naphthalic hydrazide.³⁸ However, its detection is a function of the hydrogen peroxide concentration. At high hydrogen peroxide concentrations it is not detectable. Rate studies are difficult to do and indicate that the steps prior to the production of the diazaquinone are rate determining. The few facts that are available support the hypothesis that azaquinones are intermediates in the light-producing oxidation of the hydrazides.

The intermediates proposed in Eqs. (5c) and (5d), instead of being formed directly, could also be thought of as arising from reaction of the azaquinone with hydroperoxide ion or superoxide ion [Eq. (7)]. Evidence



has been presented that superoxide may be involved in the reaction of luminol with base and oxygen in aqueous media, since superoxide dismutase quenches the chemiluminescent reaction.³⁹

³⁵ K.-D. Gundermann, *Angew. Chem.* **80**, 494 (1968).

³⁶ E. H. White, E. G. Nash, D. R. Roberts, and O. C. Zafiriou, *J. Am. Chem. Soc.* **90**, 5932 (1968).

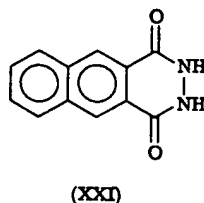
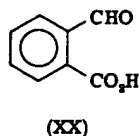
³⁷ K.-D. Gundermann, in "Chemiluminescence and Bioluminescence" (M. J. Cormier, D. M. Hercules, and J. Lee, eds.), p. 209. Plenum, New York, 1973.

³⁸ C. Lee, unpublished results, 1974.

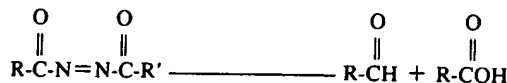
³⁹ E. K. Hodgson and I. Fridovich, *Photochem. Photobiol.* **18**, 451 (1973).

Singlet oxygen, produced via dye sensitization, has been shown to cause luminol chemiluminescence in aqueous base.⁴⁰⁻⁴² Singlet oxygen produced by laser irradiation produces only very weak chemiluminescence from luminol, and 3-aminophthalate is not the major product. It has been suggested that emission arising in the dye sensitization experiments is due to radicals being produced concurrently with singlet O_2 .⁴²

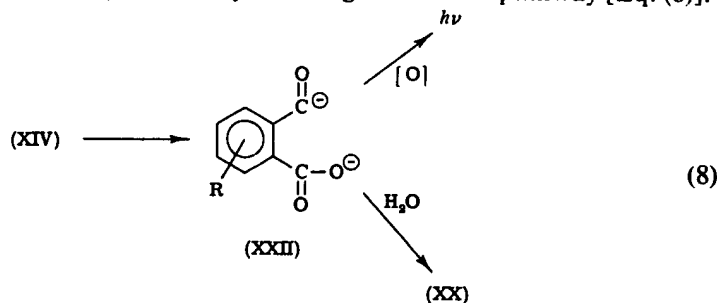
The oxidation of hydrazides under mild conditions can produce products other than phthalate ion. Reaction of phthalic hydrazide with base and potassium ferricyanide or hydrogen peroxide-ammonium hydroxide-copper has been shown to produce phthalaldehydic acid, (XX), as well as phthalate ion itself.⁴³ Oxidation of the naphthalic hydrazide, (XXI), with



basic hypochlorite mixtures also produces the corresponding acid aldehyde.³⁸ The formation of the aldehyde is not surprising, since diacylimides are known to react with bases to produce one equivalent of acid and one of aldehyde [Eq. (8)].⁴⁴



The yield of aldehyde in the hydrazide reactions decreases at the higher hydrogen peroxide concentrations that lead to enhanced light emission. Thus, the aldehyde may be a product of a dark reaction and the interception of species (XXII) may be directly on the light-emission pathway [Eq. (8)].



⁴⁰ K. Kuschner and T. Kuwana, *Chem. Commun.* 1969, 193 (1969).

⁴¹ I. B. C. Matheson and J. Lee, *Photochem. Photobiol.* 12, 9 (1970).

⁴² I. B. C. Matheson and J. Lee, *Photochem. Photobiol.* 24, 605 (1976).

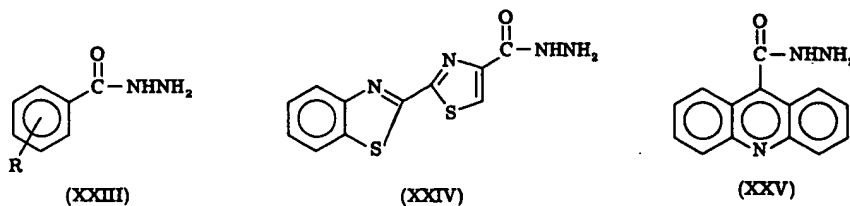
⁴³ T. Huang, unpublished results, 1972.

⁴⁴ C. Niemann and J. T. Hays, *J. Am. Chem. Soc.* 74, 5796 (1952).

In summary, it appears that dianion (XIV) is involved in aprotic oxidations, the radical anion (XV) in protic ones, and the azaquinones very probably are involved in the light pathway.

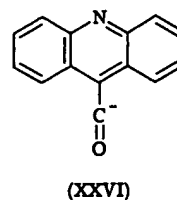
Monoacylhydrazides

Noncyclic monoacylhydrazides, such as compounds (XXIII)–(XXV), have been reported to be chemiluminescent.^{45,46} The oxidizing systems used are the same as those used for the cyclic hydrazides. The



monoacylhydrazides are moderately chemiluminescent compounds; compounds (XXIV) and (XXV) have $\Phi_{\text{Cl}} = 0.003$ (in comparison, the value for luminol is 0.01).

The pathway leading to light emission appears to be different from that of luminol and the cyclic hydrazides,⁴⁷ and thus these compounds are not good models for the luminol chemiluminescence. The emitter in the case of compound (XXV) corresponds to the fluorescence of acridone and its anion. Thus the most efficient light-producing reaction requires a decarbonylation or decarboxylation. One crucial intermediate would be the acyl anion (XXVI), which could lose carbon monoxide and react with oxygen to

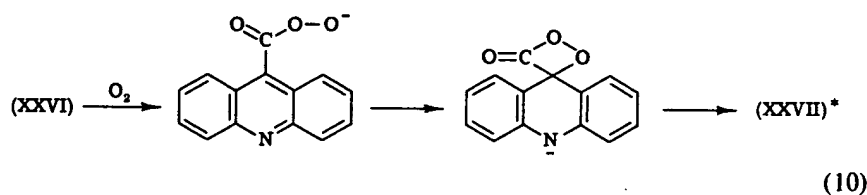
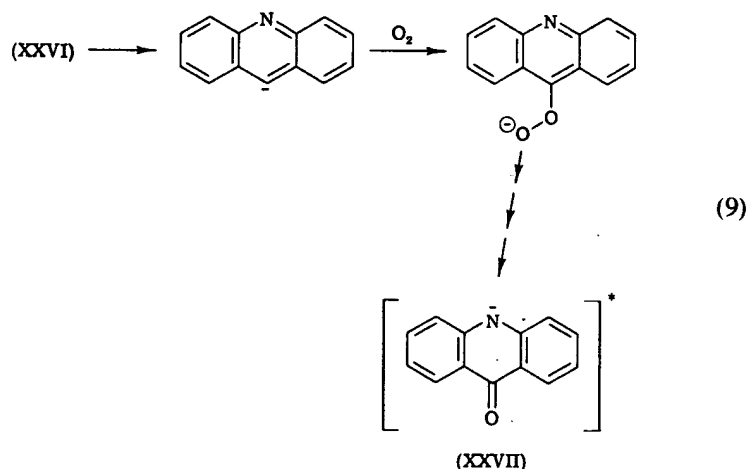


⁴⁵ E. H. White, M. M. Bursey, D. F. Roswell, and J. H. M. Hill, *J. Org. Chem.* **32**, 1198 (1967).

⁴⁶ E. Rapaport, M. W. Cass, and E. H. White, *J. Am. Chem. Soc.* **94**, 3153 (1972).

⁴⁷ In an earlier report⁴⁶ it was erroneously proposed that the mechanism of oxidation of (XXIV) and (XXV) were the same as for the cyclic hydrazides, and that the corresponding carboxylate ion was the light emitter.

form the excited state of acridone anion^{48,49} [(XXVII), Eq. (9)]. Alternatively, the acyl anion could react with oxygen and then decarboxylate via a dioxetane intermediate with formation of an excited state⁵⁰⁻⁵² [Eq. (10)]. Note that acridine carboxylic acid is only weakly fluorescent and that it may be formed via the luminol pathway also, but the low quantum yield to be expected precludes detection of this pathway.⁴⁷



The decarbonylation (decarboxylation) pathway does not appear to be operative in the case of the cyclic hydrazides, since naphthalic hydrazide, (XXI), yields naphthalate excited states, but not those of 3-hydroxyl-2-naphthoic acid.

⁴⁸ G. A. Russell and E. G. Janzen, *J. Am. Chem. Soc.* **84**, 4153 (1962).

⁴⁹ G. A. Russell and A. G. Bemis, *J. Am. Chem. Soc.* **88**, 5491 (1966).

⁵⁰ F. McCapra, *Acc. Chem. Res.* **9**, 201 (1976).

⁵¹ N. J. Turro, P. Lechtken, N. E. Schore, G. Schuster, H. C. Steinmetzer, and A. Yekta, *Acc. Chem. Res.* **7**, 97 (1974).

⁵² E. H. White, J. D. Miano, C. J. Watkins, and E. Breaux, *Angew. Chem., Int. Ed.* **13**, 229 (1974).